REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
12/01/2015	Final	Jul 2012 - Sep 2013

4. TITLE AND SUBTITLE
Determining the molecular and genetic basis for diabetes in Navy bottlenose dolphins (Tursiops truncatus)

5a. CONTRACT NUMBER
NA

5b. GRANT NUMBER
N000141210617

5c. PROGRAM ELEMENT NUMBER
NA

6. AUTHOR(S)
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5d. PROJECT NUMBER
NA

5e. TASK NUMBER
NA

5f. WORK UNIT NUMBER
NA

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
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8. PERFORMING ORGANIZATION REPORT NUMBER
NA

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Office of Naval Research
875 North Randolph Street
Arlington, VA 22203-1995

10. SPONSOR/MONITOR'S ACRONYM(S)
ONR

11. SPONSOR/MONITOR'S REPORT NUMBER(S)
NA

12. DISTRIBUTION/AVAILABILITY STATEMENT
Unlimited

13. SUPPLEMENTARY NOTES
NA

14. ABSTRACT
CREB-ZF functions as a transcriptional repressor, at least in terms of its effects on gluconeogenic genes. Efforts are underway to determine whether CREB-ZF directly interferes with activation of the cAMP-responsive factor CREB or the CRTC family of CREB coactivators. CREB-ZF has been reported to modulate the unfolded protein response (UPR) although the underlying mechanism is unclear. Because it contains a leucine zipper DNA binding domain, CREB-ZF would be expected to compete for binding to CREB binding sites. We will test whether CREB-ZF displaces CREB and CRTC2 from gluconeogenic promoters and thereby reduces hepatic glucose production.

15. SUBJECT TERMS
Gluconeogenesis, CREB ZF, Fasting, Diabetes

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			Marc R. Montminy
					19b. TELEPHONE NUMBER *(Include area code)*
U	U	U	UU	11	858-453-4100 x1394

ONR Technical / Final Report:
July 2012 - September 2013

PI Name: Marc Montminy, M.D., Ph.D.
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ONR Award Number: N000141210617
Award Title: Determining the molecular and genetic basis for diabetes in Navy bottlenose dolphins (Tursiops truncates)

Contents

I. Scientific and Technical Objectives

Navy bottlenose dolphins (*Tursiops truncatus*) are susceptible to insulin resistance, and they appear to have a switch that turns a diabetes-like state on and off.[1,2] While this metabolic state was initially thought to be normal for dolphins, recent studies have discovered that dolphins are susceptible to a number of chronic conditions associated with insulin resistance, including fatty liver disease, iron overload, urate nephrolithiasis, hyperlipidemia and chronic inflammation.[3-6] These chronic conditions are present among Navy dolphins, and the study's primary hypothesis is that there is a molecular and genetic basis for insulin resistance in Navy dolphins. The scientific objective of this study was to begin characterizing the molecular and genetic basis of the gluconeogenic program in dolphins, with a focus on the genes and promoters for glucose-6-phosphatase (G6Pase) and PEPCK, two rate-limiting enzymes for gluconeogensis in mammals. The technical objectives of this 1-year study were as follows:

- Amplify PCR genomic fragments corresponding to promoters for dolphin G6Pase and PEPCK genes
- Construct G6Pase and PEPCK promoter luciferase reporter constructs
- Use RT-PCR to obtain cDNAs for dolphin CREB-ZF, a member of the basic leucine zipper domain (bZIP) family of transcription factors with homology to cAMP response element binding protein (CREB)
- Perform transient transfection assays to test effects of CREB ZF over-expression on gluconeogenic gene expression in response to fasting signals

II. Approach

A. Amplify PCR genomic fragments corresponding to promoters for dolphin G6Pase and PEPCK genes. The publically available partial dolphin genome from Baylor College of Medicine, based upon DNA from a Navy dolphin, was used to identify genomic fragments corresponding to promoters for dolphin G6Pase and PEPCK genes. Polymerase chain reactions (PCR) were used to amplify these fragments. To assess how well they were conserved, these dolphin gene sequences were compared to those from humans, mice, and rats. Targeted binding sites, including TATA boxes, included those for the transcription factors, forkhead box O1 binding protein (FOXO1) and CREB.

B. Construct G6Pase and PEPCK promoter luciferase reporter constructs. Luciferase was used to assess successful transcriptional activity of dolphin G6Pase and PEPCK promoters. Cellular levels of cAMP were raised with forskolin (FSK) to mimic the fasting state, and relative luciferase activity was measured at 0, 2, 4, and 6 hours.

C. Obtain cDNAs for dolphin CREB-ZF. While searching for the dolphin CREB gene, dolphin CREB-ZF was identified as a potential transcription factor. CREB-ZF is a member of the basic leucine zipper domain (bZIP) family of transcription factors with homology to CREB. RT-PCR was used to obtain cDNAs for dolphin CREB-ZF.

D. Test effects of CREB-ZF over-expression on gluconeogenic gene expression. Transient transfection assays were used to test the effects of CREB-ZP on gluconeogenic gene expression in response to fasting signals (cAMP).

III. Concise Accomplishments

A. Amplify PCR genomic fragments corresponding to promoters for dolphin G6Pase and PEPCK genes
- Basic mechanisms for activation of gluconeogenic genes are conserved in dolphins.

B. Construct G6Pase and PEPCK promoter luciferase reporter constructs
- Dolphin PEPCK transcription increased in the face of increasing cAMP, supporting that this enzyme induces gluconeogenesis during the fasting state.

C. Obtain cDNAs for dolphin CREB-ZF
- Dolphin CREB-ZF, closely related to CREB, was identified as a potential transcription factor (genetic switch).

D. Test effects of CREB-ZF over-expression on gluconeogenic gene expression
- Dolphin CREB-ZF is a novel, negative regulator of gluconeogenesis.

Over the last few months of this grant, we continued our efforts to address the mechanism by which the transcription factor CREB –ZF regulates hepatic gluconeogenesis. Pilot studies revealed that CREB-ZF functions as a transcriptional repressor, at least in terms of its effects on gluconeogenic genes. Efforts are underway to determine whether CREB-ZF directly interferes with activation of the cAMP-responsive factor CREB or the CRTC family of CREB coactivators.

CREB-ZF has been reported to modulate the unfolded protein response (UPR) although the underlying mechanism is unclear. Because it contains a leucine zipper DNA binding domain, CREB-ZF would be expected to compete for binding to CREB binding sites. We will test whether CREB-ZF displaces CREB and CRTC2 from gluconeogenic promoters and thereby reduces hepatic glucose production. In future work, we will obtain full-length clones for dolphin CREB-ZF and test the effect of CREB-ZF on CREB/CRTC2 occupancy and recruitment.

IV. Expanded Accomplishments

A. Amplify PCR genomic fragments corresponding to promoters for dolphin G6Pase and PEPCK genes. Figures 1 and 2 demonstrate the homology of these dolphin genes with those in human, mouse, and rat. FOXO1 and CREB binding sites are conserved in dolphin G6Pase promoter compared to human, mouse, and rat genes. CREB binding site is conserved on dolphin PEPCK promoter compared to human, mouse, and rate genes.

Figure 1. FOXO and CREB binding sites are conserved in dolphin G6Pase promoter compared to human, mouse, and rat genes.


Foxo Binding Site
Foxo Binding Site
CREB Binding Site
TATA Box
human
dolphin
mouse
rat
CTGTTTTTGTGTGCCTGTTTTTCTATTTTACGTAAATCACCCTGAACATGTTTGCATCAA
CTGTTTTTCTGTGCCTGTTTTCCTATTTTACGTAAATCACACTGAACACGTTTGCATCAA
CTGTTTTTGTGTGCCTGTTTTGCTATTTTACGTAAATCACCCTGAACATGTTTGCATCAA
CTGTTTTTGTGTGCCTGTTTTGCTATTTTACGTAAATCACCCTGAACATGTTTGCATCAA
CCTACTGGTGATGCACCTTTGATCAATACATTTTAGACAAACGTGGTTTTT-GAGTCCAA
CCTACTGGTGATGCACCTTTGATCAATAGATTTTAGACAAAAGCGGTTTTT-GAGTCCAA
CCTACTGATGATGCACCTTTGATCAATAGATTTTAGACAAAAGTGGTTTTTTGAGTCCAA
CCTACTGATGATGCACCTTTGATCAATAGATTTTAGACAAAAGTGTTTTTTTGAGTCCAA
AGATCAGGGCTGGGTTGACCTGAATACTGGATACAGGGCATATAAAACAGGGGCAAGGCA
AGATCAGGGCTGGGTTGACCTGCAGACTGGATACAGAGTGTATAAAACAGAGGCAAGACA
AGATCAGGGCTGGATTGACCTACAGACTGAATCCAGGGCATATAAAACAGGGGCAAGGCA
AGATCAGGGCTAGGTTGACCTACAGACTGAATCCAGGGCATATAAAAT--GGGCAAGGCA

Figure 2. CREB binding site is conserved in dolphin PEPCK promoter compared to human, mouse, and rat genes.

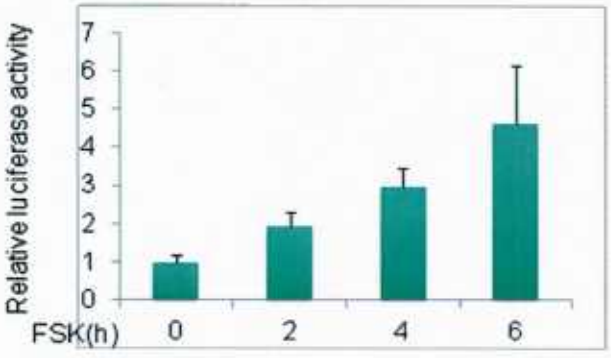
CREB Binding Site

```
human      CAAAGCATAACTGACCCTGGCCGTGATCCAGAGACCTGCCCCCTGACGTCAGTGGCGAGC
dolphin    CAAAGCATAACTGACCGTGGCCGTGATCCAAAGACCAGCCCC-TGACGTCAGAGATGAGC
mouse      CAAACCGTG-CTGACCATGGCTATGATCCAAAGGCCTGCCCC-TTACGTCAGAGGCGAGC
rat        CAAACCGTG-CTGACCATGGCTATGATCCAAAGGCCGGCCCC-TTACGTCAGAGGCGAGC
           **** * *  ****** ****  ******* ** ** ***** * ******* *  ****

human      CTCCCTGGGTGCAGCTGAGGGGCAGGGC----TATTCTTTTCCACAGTATTTAAAGCTGG
dolphin    CTCCTTGGGTGTGGCTGAGGGGCTGGAC----CTCTGTCCTTCATGGAATTTAAAGCCAG
mouse      CTCC--GGGTCCAGCTGAGGGGCAGGGCTGTCCTCCCTTCTATATAGTATTTAAAGCAAG
rat        CTCC--AGGTCCAGCTGAGGGGCAGGGCTGTCCTCCCTTCTGTATACTATTTAAAGCGAG
           ****   ***   ********** ** *         *  *  *    *********  *
```

B. Construct G6Pase and PEPCK promoter luciferase reporter constructs. PEPCK luciferase reporter was successfully induced in response to cAMP agonist, FSK. Figure 3 demonstrates increasing luciferase activity over 6 hours. This demonstrated that the dolphin PEPCK promoter, in the face of a mimicked fasting setting, behaved as expected in other mammals.

Figure 3. Induction of PEPCK luciferase reporter in response to cAMP agonist (FSK)



C. Obtain cDNAs for dolphin CREB-ZF. Dolphin CREB-ZF had a basic leucine zipper (bZIP) domain, indicating that it is a transcription factor, or switch that may regulate gene activity. Dolphin CREB-ZF had distinct regions that were not homologous to CREB-ZF in mice, supporting that it may be a different construct.

Figure 4. Dolphin CREB-ZF sequence, including a bZIP domain, compared to mouse.

```
mouse          MRHSLTKLLAASGRDFPSRRDSREPPATRAPPREPSGAAAGAE-TPRPGSPDREQPHGDG
dolphin        MRHSLTKLLAASGSDSPTRSESPAPVATCSLPPDLTRAAAEDEGTAAAGSPGRKQPRGD-
               ************ * *:* :*  * ** : * : : ***  * *. .***.*:**:**

mouse          DGGEPEARSGSRGSVAVRAPAPSPLKMEEEEEDAIAMVPKEEPEDMDFLSGLELADLLDP
dolphin        -EGESDAGRGGRGIVAARAPSP-----EEMEEEAIASVPGEETEDMDFLSGLELADLLDP
                 **.:*  *.** **.***:*     ** **:*** ** **.*****************

mouse          RQPDWHLEPGLSSPGPLSSSGGGSESGGLLRGDDDDDTAAAEMQRFSDLLQRLLNGIGGC
dolphin        RQPDWHLEPGLNSPGPLSSSGGGSDSGGLWRGDDDDEAAAAEMQRFSDLLQRLLNGIGGC
               ***********.************:**** ******::**********************
                                                                  bZIP domain
mouse          SSGGDRGGGEKRRRKSPGAGGGG-ANDGNQAATKSPRKAAAAAARLNRLKKKEYVMGLES
dolphin        SSGSDSGGGEKRRRKSPGGGGGSSGNDNNQAATKSPRKAAAAAARLNRLKKKEYVMGLES
               ***.* ************.***. .**.*************************************

mouse          RVRGLAAENQELRAENRELGKRVQALQEESRYLRAVLANETGLARLLSRLSGVGLRLTTS
dolphin        RVRGLAAENQELRAENRELGKRVQALQEESRYLRAVLANETGLARLLSRLSGVGLRLTTS
               ************************************************************

mouse          LFRDSPAGDHDYALPVGKQPPEPREEDDAAGGVCLHVDKDKVSVEFCSACARKASSSLKM
dolphin        LFRDSPAGDHDYALPVGKQQQDLLEEDDSAGGVCLHVDKDKVSVEFCSACARKASSSLKM
               ******************  :  ****:*******************************
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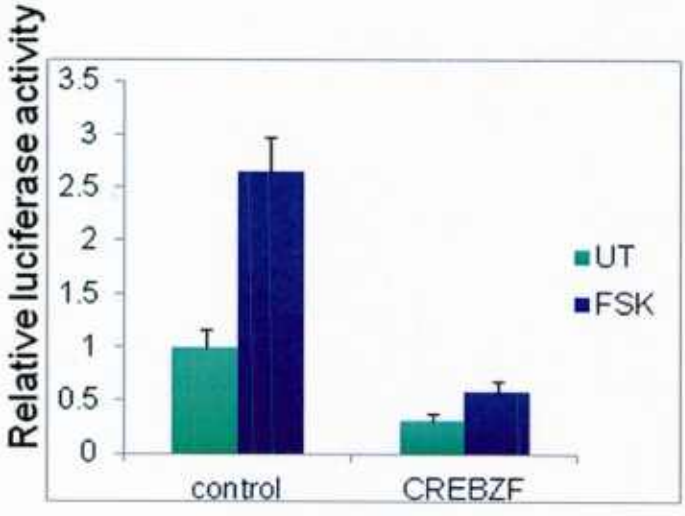
D. Effects of CREB-ZF over-expression on gluconeogenic gene expression. Dolphin CREB-ZF over-expression inhibited PEPCK promoter activity in the face of elevated cAMP (Figure 5). This is the first report of CREB-ZF involvement in metabolism. Dolphin CREB-ZF is a novel, negative regulator and potential 'off switch' for gluconeogenesis.

Figure 5. Effect of CREBZF over-expression on dolphin pck1 promoter activity in cells exposed to cAMP agonist FSK for 4 hours. UT, untreated.



V. Future Plans (new white paper)

1. Obtain cDNA clones for dolphin CREB1 using RT-PCR.

2. Continue characterizing dolphin gluconeogenic program by evaluating relevant molecules and genes in liver, muscle, and adipose tissue.

3. Test the role of CREB-ZF in modulating hepatic gluconeogenesis by adenoviral over-expression or RNAi-mediated knockdown in mouse hepatocytes and in liver.

4. Determine the mechanism by which CREB-ZF inhibits gluconeogenic gene expression. Specifically, we will test whether CREB-ZF associates with CREB1 or with CREB-cofactors such as the CRTCs.

5. Identify signals that regulate CREB-ZF expression and activity in liver. In particular, we will test effects of high fat diet feeding on CREB-ZF expression and activity. Does high fat diet feeding increase gluconeogenic gene expression by reducing CREB-ZF expression?

6. Test relative activities of mouse and dolphin CREB-ZF proteins. Could disparities in fasting glucose production between dolphins and other mammals reflect differences in respective CREB-ZF proteins?

VI. Major Problems/Issues

Lack of a full dolphin genome sequence led to difficulties in finding the transcription factor, CREB.

VII. Technology Transfer

Due to well conserved mechanisms for gluconeogenesis, drugs like metformin, which down-regulate gluconeogenic gene expression, may be effective in controlling fasting glucose in Navy dolphins. The data also suggest that increasing expression of the inhibitor CREB-ZF in hepatocytes may reduce gluconeogenic gene expression and ciruculating blood glucose levels in Navy dolphins.

VIII. Foreign Collaborations and Supported Foreign Nationals

None

IX. Productivity

A. Refereed journal articles

None

B. Non-refereed significant publications

None

C. Books and chapters

No books or chapters have been generated in relation to this project.

D. Technical reports

No technical reports have been generated in relation to this project.

E. Patents

No patents have been generated in relation to this project.

F. Awards/honors

No awards or honors have been generated in relation to this project.

X. Award Participants

The following non-military personnel received salary support from this ONR award during the reporting period: Bing Luan, Ph.D.

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